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The discovery of SycO highlights a new function for type III secretion effector chaperones

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Bacterial injectisomes deliver effector proteins straight into the cytosol of eukaryotic cells (type III secretion, T3S). Many effectors are associated with a specific chaperone that remains inside the bacterium when the effector is delivered. The structure of such chaperones and the way they interact with their substrate is well characterized but their main function remains elusive. Here, we describe and characterize SycO, a new chaperone for the *Yersinia* effector kinase YopO. The chaperone-binding domain (CBD) within YopO coincides with the membrane localization domain (MLD) targeting YopO to the host cell membrane. The CBD/MLD causes intrabacterial YopO insolubility and the binding of SycO prevents this insolubility but not folding and activity of the kinase. Similarly, SycE masks the MLD of YopE and SycT covers an aggregation-prone domain of YopT, presumably corresponding to its MLD. Thus, SycO, SycE and most likely SycT mask, inside the bacterium, a domain needed for proper localization of their cognate effector in the host cell. We propose that covering an MLD might be an essential function of T3S effector chaperones.

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Introduction

Type III secretion (T3S) allows pathogenic and symbiotic Gram-negative bacteria to deliver bacterial effector proteins straight into the cytosol of an eukaryotic host cell (Cornelis and Wolf-Watz, 1997). Translocation of the effectors is a very rapid process (Schlumberger *et al*, 2005) triggered by host cell contact (Pettersson *et al*, 1996) and achieved in one step by a sophisticated nanomachine called the injectisome or needle complex (Kubori *et al*, 1998; Mueller *et al*, 2005). The assembly and operation of the injectisome involves small (12–18 kDa) chaperones that remain in the bacterial cytosol (Wattiau and Cornelis, 1993; Menard *et al*, 1994; Feldman and Cornelis, 2003; Parsot *et al*, 2003; Ghosh, 2004). These chaperones represent a special family of chaperones found only in T3S systems. Some of these chaperones are involved in the assembly of the injectisome (class III) or the translocation pore (class II), whereas others are ancillary to effectors (class I). Class-I chaperones are acidic (p*I*: 4–5), usually dimeric, proteins, which bind their cognate effector within their first 100 amino acids, just downstream of the short N-terminal secretion signal. They are often, but not always, encoded next to the gene encoding their partner protein. They present a low sequence similarity, but their structure is quite well conserved. Several three-dimensional structures of T3S chaperones have been solved during the last years, showing that they all adopt a similar fold consisting of five β-strands and three α-helices (Birtalan and Ghosh, 2001; Luo *et al*, 2001; Stebbins and Galan, 2001; Evdokimov *et al*, 2002; Trame and McKay, 2003; Phan *et al*, 2004; van Eerde *et al*, 2004; Buttner *et al*, 2005; Locher *et al*, 2005). Structures of chaperones in complex with their effector proteins have shown that the chaperone-binding domain (CBD) of effectors wraps around the homodimers in an extended, horseshoe-like conformation with some secondary structure organization in α-helical structures (Stebbins and Galan, 2001; Birtalan *et al*, 2002; Phan *et al*, 2004). Chaperones are removed from effectors, before their translocation into host cells, by the ATPase that is part of the injectisome (Woestyn *et al*, 1994; Akeda and Galan, 2005). Although some chaperones of T3S effectors have been intensively studied and characterized, their essential function remains a matter of controversy. Some studies suggest that chaperones could be three-dimensional targeting factors or even that they play a role in the setting of a secretion hierarchy (Boyd *et al*, 2000; Birtalan *et al*, 2002). Other observations suggest that they are required for the storage of effectors in the bacterial cytosol before secretion (Page *et al*, 2002) and even that they prevent folding during storage (Stebbins and Galan, 2001; Page *et al*, 2002).

The Ysc (for Yop Secretion) T3S system allows pathogenic *Yersinia* (*Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica*) to escape the innate immune defenses. It is encoded on a 70-kb virulence plasmid, called pYV in *Y. enterocolitica*, and it injects six effector proteins, called Yops, into the cytosol of macrophages. YopH, YopE, YopT and YopO disturb the cytoskeleton dynamics and inhibit phagocytosis (Cornelis, 2002). Three of these Yops, namely YopE, YopH and YopT, have a dedicated chaperone called SycE (Wattiau and Cornelis, 1993; Birtalan and Ghosh, 2001), SycH (Wattiau *et al*, 1994; Phan *et al*, 2004) and SycT (Iriarte and Cornelis, 1998; Buttner *et al*, 2005; Locher *et al*, 2005), respectively. No chaperone has been described so far for YopO (called YpkA in *Y. pestis* and

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Y. pseudotuberculosis), a broad-spectrum serine threonine kinase (Galyov *et al*, 1993) that becomes activated upon contact with actin (Juris *et al*, 2000). It is localized to the plasma membrane of the target cell (Hakansson *et al*, 1996; Dukuzumuremyi *et al*, 2000) and it interacts with Rho and Rac (Barz *et al*, 2000) but its physiological target is still unknown. The gene encoding YopO (YpkA) is part of an operon encoding also the effector YopP (Cornelis *et al*, 1987; Galyov *et al*, 1994; Iriarte and Cornelis, 1999) (YopJ in *Y. pestis* and *Y. pseudotuberculosis*), which interrupts pro-inflammatory signalling cascades and leads macrophages to apoptosis (Mills *et al*, 1997; Zhou *et al*, 2005). The first open reading frame of the operon, *orf155*, encodes a protein that presents all the characteristics of a T3S chaperone (Iriarte and Cornelis, 1999), but its role could not be demonstrated so far (Trulzsch *et al*, 2003).

Here, we demonstrate that *orf155* indeed encodes a chaperone for YopO and we called it SycO. We show that SycO prevents the intrabacterial aggregation of YopO by covering a membrane localization domain (MLD). As many T3S effectors, which have a chaperone, have also been shown to be membrane associated, these observations suggest that covering a MLD could be the primary function of the T3S effector chaperones.

Results

orf155 encodes a protein that has the characteristics of chaperones of effector proteins

In the three pathogenic *Yersinia* species, the operon encoding YopO (YpkA) and YopP (YopJ) contains a small open reading frame (*orf*) encoding a 142- (*Y. pestis* KIM) to 155- (*Y. enterocolitica* E40) residue acidic protein (Galyov *et al*, 1994; Iriarte and Cornelis, 1999) (Supplementary Figure S1). Upstream of residue 133, the three proteins are 98% identical. Programs searching sequence similarities and fold recognition revealed low but significant similarities between Orf155 and various T3S class-I chaperones (Supplementary Figure S1). Based on this, a three-dimensional structure, very similar to that of class-I chaperones, could be derived (Supplementary Figure S1 and Supplementary data 1).

orf155 encodes SycO, the chaperone of YopO

The entire *orf155* was deleted from the pYV plasmid of *Y. enterocolitica* E40(pYV40) giving E40(pML4001) (Δ*orf155* mutant). Yop secretion was triggered by chelating Ca^{2+} ions (Cornelis *et al*, 1987). Under standard induction conditions (4 h), all the Yops were detected in the culture supernatant (not shown). However, when the time allowed for secretion was reduced, there was less YopO (YpkA) in the culture supernatant of mutant bacteria than in the culture supernatant of wild-type (wt) bacteria (Figure 1A). No other Yop, including YopP, encoded downstream of *orf155* and *yopO* was affected, indicating that the mutation was non-polar (data not shown). As known for the other T3S chaperones, SycO itself was not secreted (data not shown).

We then monitored translocation of YopO into macrophages during the infection of a cell culture, using the adenylate cyclase (Cya) reporter method (Sory and Cornelis, 1994). We introduced plasmid pCD10, encoding the first 143 amino acids of YopO fused to Cya, into wt *Y. enterocolitica* E40 as well as into the Δ*orf155* mutant and various control strains

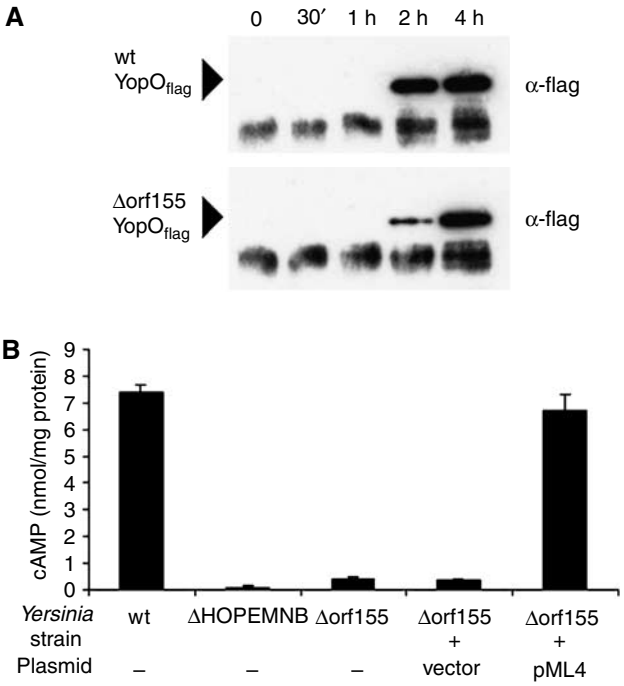

Figure 1 The Δ*orf155* mutant is impaired in secretion and translocation of YopO. (**A**) Plasmid pISO56 encoding YopO$_{flag}$ was introduced into wt E40(pYV40) bacteria and into Δ*orf155* mutant bacteria E40(pML4001). Yop secretion was induced by incubating at 37°C in a medium deprived of Ca^{2+} ions. Samples of the supernatant were taken at different time points after shift of the culture from 28 to 37°C. YopO$_{flag}$ was detected by immunoblot. Top, wt; bottom, Δ*orf155* mutant. The lower background band shows that an equivalent amount of supernatant has been loaded in each lane. (**B**) Translocation of the YopO-Cya reporter into infected J774 macrophages. Plasmid pCD10 encoding YopO$_{143}$-Cya was introduced into E40(pYV40) wt bacteria, into Δ*orf155* knockout bacteria E40(pML4001) and into the translocator-deficient E40(pCNK4008) (called ΔHOPEMNB). The low-copy plasmid pML4 encoding *sycO* complemented the Δ*orf155* mutation, whereas the vector alone did not. Cellular levels of cAMP were assayed after infection.

and we infected cultured J774 macrophages. Cells that were infected by wt bacteria accumulated cAMP. In contrast, cells infected with the Δ*orf155* mutant did not produce more cAMP than cells infected with translocation-deficient bacteria ΔHOPEMNB (Figure 1B). Complementation of Δ*orf155* mutant bacteria with plasmid pML4 restored translocation of YopO up to the wt level (Figure 1B). These experiments showed that the product of *orf155* is required for translocation of YopO and hence, given its similarity to Syc chaperones, it was tentatively called SycO.

To ascertain that translocation of YopP was not dependent on SycO, we monitored apoptosis of macrophages infected by wt *Y. enterocolitica* E40(pYV40) and by Δ*orf155 Y. enterocolitica* E40(pML4001). No difference could be seen between the two infected cultures (data not shown), confirming that SycO is not the chaperone of YopP, as shown earlier (Trulzsch *et al*, 2003).

SycO binds to residues 20–77 of YopO

To confirm that SycO is the chaperone of YopO, we tested whether the two proteins interact and how. The *sycO-yopO* genes were coexpressed in *Escherichia coli* BL21 and SycO was co-purified with YopO$_{his}$ (data not shown) suggesting

that the two proteins form a complex when synthesized together in *E. coli*. To ascertain this observation, a sample of co-purified proteins was loaded on an analytical gel filtration column. YopO and SycO eluted together around fraction 9 (Figure 2A), whereas purified SycO loaded alone eluted around fraction 14 (Figure 2B). To localize the CBD of YopO, three different YopO-Cya hybrid proteins were produced in the multi-effector knockout *Y. enterocolitica* ΔHOPEMT together with GST-SycO. $YopO_{143}$-Cya and $YopO_{77}$-Cya co-purified with SycO on glutathione Sepharose, whereas $YopE_{20}$-Cya did not (not shown). These experiments led to the conclusion that the CBD of SycO is localized within the 77 N-terminal residues of YopO. As SycE binds to residues 15–75 of YopE (Woestyn *et al*, 1996; Birtalan *et al*, 2002) and SycH binds to residues 20–70 of YopH (Woestyn *et al*, 1996), we made the assumption that SycO could also bind immediately after the secretion signal of YopO. To test this hypothesis, we constructed a GST-YopO hybrid, a variant where residues 20–77 of YopO were deleted (GST-$YopO_{\Delta 22-77}$) and a hybrid containing only residues 20–77 of YopO (GST-$YopO_{20-77}$). All three hybrid proteins were expressed in parallel in *Y. enterocolitica* E40. SycO was pulled down together with GST-YopO and with GST-$YopO_{20-77}$ but not with GST-$YopO_{\Delta 20-77}$ (Figure 2C). This indicated that residues 20–77 of YopO include the core of the CBD.

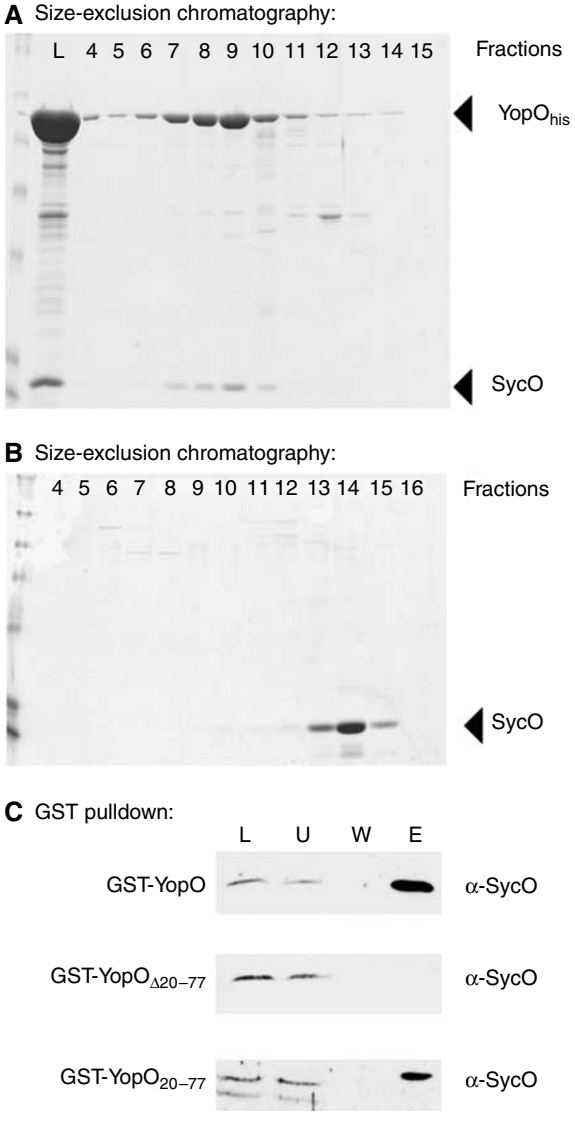

Figure 2 Characterization of the YopO–SycO interaction. (**A**) $YopO_{his}$ and SycO encoded by pML9 were synthesized in *E. coli* BL21 and co-purified on Ni^{2+} Sepharose. The purified complex (L) was loaded onto a Superdex^{TM} 200 gel filtration column and the eluted fractions (4–15) were analyzed by Coomassie-stained SDS–PAGE. $YopO_{his}$ and SycO eluted together around fraction 9. (**B**) Purified SycO protein (see Supplementary data 4), loaded on the same column, eluted around fraction 14. (**C**) Plasmids pML10, pML11 and pML17 encoding GST-YopO, GST-$YopO_{\Delta 20-77}$ and GST-$YopO_{20-77}$, respectively, were introduced into *Y. enterocolitica* E40 wt producing SycO from the pYV40 plasmid. GST hybrid proteins were pulled down from crude extracts, analyzed by SDS–PAGE and the presence of SycO was monitored by immunoblotting. L, lysate; U, unbound; W, wash; E, eluted.

We then investigated the stoichiometry of the YopO–SycO complex and observed that SycO dimerizes and binds YopO as a dimer (Supplementary data 2), as known for the other class-I chaperones.

We finally compared the CBD of YopO to that of YopE *in silico* and found that they share a similar secondary structure, meaning that the YopO CBD is likely to wrap around the dimer of SycO as the YopE CBD wraps around the dimer of SycE (Supplementary data 3).

Binding of SycO to YopO does not prevent folding of the whole protein

It has been proposed that class-I chaperones could keep the effectors in an unfolded secretion competent state (Stebbins and Galan, 2001). To test this hypothesis for YopO, we monitored the autophosphorylating activity of the purified kinase (Galyov *et al*, 1993), with and without the CBD. As YopO was poorly soluble without its chaperone but was more soluble as a GST fusion or without the CBD, we compared the kinase activity of GST-YopO to that of $YopO_{\Delta 20-77}$ and YopO-$(SycO)_2$. As a negative control, we engineered an inactive GST-YopO hybrid with the D267A substitution in the catalytic site (Juris *et al*, 2000). As expected, the kinase activity was dependent on the presence of actin (Juris *et al*, 2000) (Figure 3). Importantly, $YopO_{\Delta 20-77}$ was at least as active as GST-YopO (Figure 3), showing that the CBD is not needed for the catalytic activity of YopO. In addition, the YopO-$(SycO)_2$ complex turned out to be as active as GST-YopO, showing that SycO binding does not prevent folding of the whole protein. Interestingly, SycO itself was phosphorylated. This has no physiological relevance, as chaperones are not translocated into host cells (Wattiau *et al*, 1994), but it confirms the broad spectrum activity of YopO (Juris *et al*, 2000).

$YopO_{\Delta CBD}$ can be exported by the Ysc T3S system and translocated into host cells

To further investigate the role of SycO, we removed the CBD within YopO and first tested the consequences in terms of secretion triggered by Ca^{2+} chelation. We compared secretion of $YopO_{flag}$ and $YopO_{\Delta 20-77flag}$ after 3 h of incubation at 37°C in the presence and absence of SycO. As expected, the absence of SycO had a negative effect on secretion of YopO but not on secretion of $YopO_{\Delta 20-77}$. Unexpectedly, there was more $YopO_{\Delta 20-77flag}$ than $YopO_{flag}$ in the supernatant and in the lysate of wt bacteria (Figure 4A). However, it has been shown in *Salmonella* that the chaperone prevents the effectors SptP and SopE to be secreted by the flagellum rather than by the injectisome (Lee and Galan, 2004). To ensure that

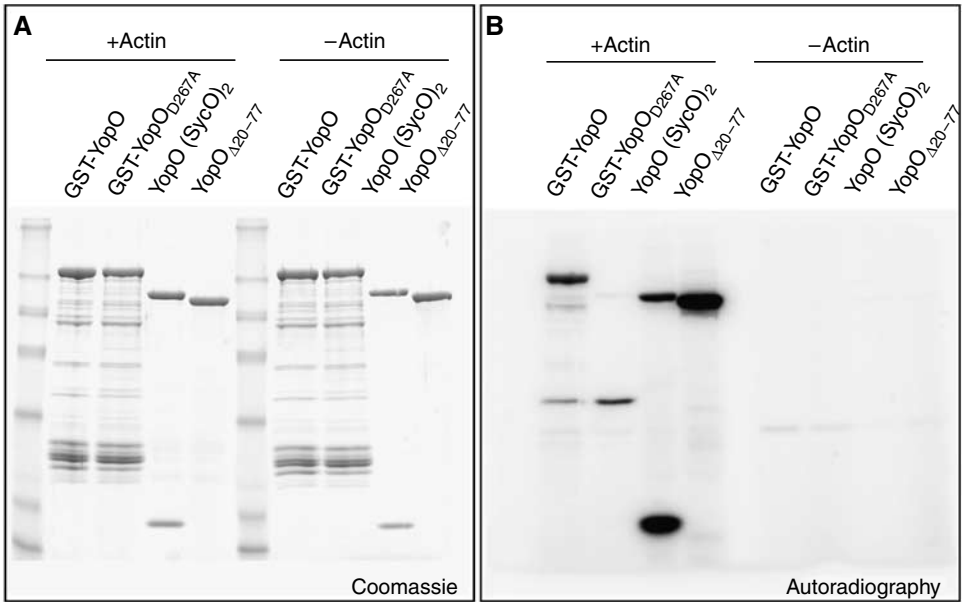

Figure 3 Binding of SycO to YopO does not prevent the catalytic activity of YopO. GST-YopO, GST-YopO$_{D267A}$, YopO(SycO)$_2$ and YopO$_{\Delta 20-77}$, expressed in *E. coli* BL21 from plasmids pML10, pML12, pML10 + pML15 and pML11 respectively, were semipurified (Supplementary data 4), incubated with [γ-^{32}P]ATP in the presence and absence of actin and analyzed by Coomassie-stained SDS–PAGE (**A**) and autoradiography (**B**).

YopO$_{\Delta 20-77flag}$ was indeed secreted by the injectisome, we monitored secretion by *Y. enterocolitica* W227(pSW2276) mutated in *yscN*, the gene encoding the ATPase energizing the T3S pathway. As shown in Figure 4A, the protein was not secreted, ruling out secretion by the flagellum. These results show that, in the absence of SycO, YopO is exported better without its CBD. This suggests that the CBD itself creates the need for SycO.

As a protein that is secreted is not necessarily delivered into cells (Figure 1), we removed the CBD from the YopO$_{143}$-Cya reporter protein and monitored translocation. Again, the observations on *in vitro* secretion and translocation were somewhat different. YopO$_{143\Delta 20-77}$-Cya was efficiently delivered into cells by ΔHOPEMT bacteria (Figure 4B) but not by wt bacteria (not shown). Thus, for translocation, we can conclude that the presence of the CBD and the chaperone facilitates the delivery of the effector but targeting can occur in their absence.

SycO prevents the intrabacterial aggregation of YopO mediated by the CBD

In order to understand why the presence of the CBD reduces the amount of YopO secreted *in vitro*, we compared the solubility of YopO and YopO$_{\Delta 20-77}$ in *E. coli*. In the absence of SycO, YopO$_{\Delta 20-77his}$ was abundant and mostly soluble, whereas YopO$_{his}$ was present in very small amounts and exclusively in the insoluble fraction (Figure 5A). In the presence of SycO, there was more YopO$_{his}$ than in the absence of SycO and most of it was soluble. This suggested that YopO is less soluble than YopO$_{\Delta 20-77}$ and that SycO helps solubilizing YopO.

To confirm this observation, we purified YopO and YopO$_{\Delta 20-77}$ and the YopO-(SycO)$_2$ complex using GST tags that were cleaved off at the last stage. Again, the yield of YopO was much lower than the yield of the two others (not shown). The three preparations as well as SycO alone were analyzed by size-exclusion chromatography (Figure 5B). Whereas SycO, YopO-(SycO)$_2$ and YopO$_{\Delta 20-77}$ were eluted as expected from their size, YopO appeared in the void volume, indicating that it was aggregated. Thus, the CBD creates the need for SycO because it drastically reduces the intrabacterial solubility of YopO.

The SycO-binding domain is a cell membrane targeting domain

The previous experiments demonstrated that SycO is required because of the presence of residues 20–77 in YopO and that these residues are not involved in the catalytic activity. As YopO is targeted to the membrane of the host cell (Hakansson *et al*, 1996; Dukuzumuremyi *et al*, 2000), we tested whether the residues 20–77 would not act as an MLD. To investigate this possibility, the 3′ end of different *yopO* constructs was fused to the EGFP gene and HEK293T cells were transfected with the recombinant plasmids. Whereas EGFP was distributed in the cytosol and the nucleus, YopO-EGFP was preferentially localized at the plasma membrane, as expected. Furthermore, cells transfected with plasmid pML1, encoding YopO-EGFP, became rounded (Figure 6), indicating that the YopO-EGFP hybrid protein had kept an activity in the cell. A YopO$_{D267A}$-EGFP catalytic mutant also localized to the membrane. This showed that the catalytic activity is not required for membrane localization. In contrast, YopO$_{\Delta 20-77}$-EGFP did not localize to membranes and cells transfected with pML2, encoding YopO$_{\Delta 20-77}$-EGFP, did not round up. We concluded that the CBD was required for the membrane targeting and that this membrane localization was necessary for YopO-EGFP activity (Figure 6). To delineate more accurately the MLD, we fused residues 20–80 and 20–90 of YopO to EGFP and monitored the localization of the hybrid. Residues 20–90, but not 20–80, were sufficient to target EGFP to the membrane. There is thus a good correlation between the CBD (20–77) and the MLD (20–90).

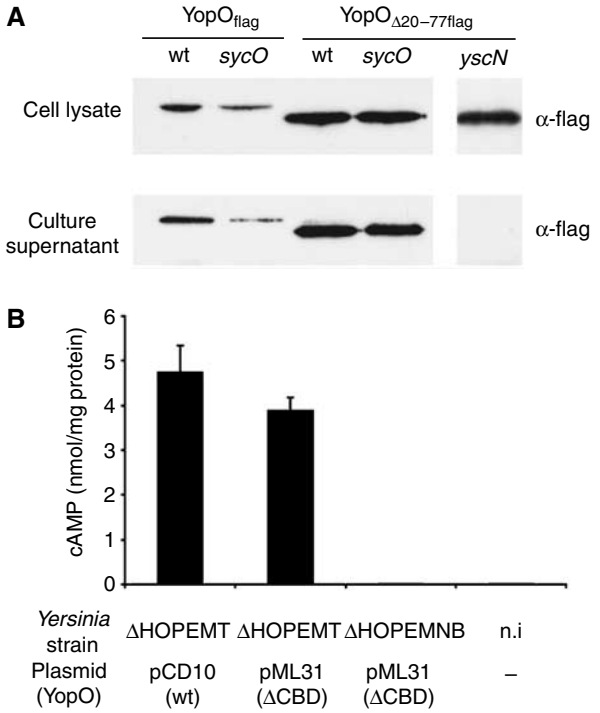

Figure 4 YopO$_{\Delta 20-77}$ can be secreted and delivered into host cells. (**A**) Plasmid pISO56 encoding YopO$_{flag}$ or pML16 encoding YopO$_{\Delta 20-77flag}$ was introduced into wt *Y. enterocolitica* E40(pYV40), Δ*sycO Y. enterocolitica* E40(pML4001) and *yscN Y. enterocolitica* E40(pSW2276). Yop secretion was triggered by Ca^{2+} chelation for 3 h and the supernatants were analyzed by Western blotting. (**B**) Translocation of the YopO$_{143\Delta 20-77}$-Cya reporter into infected J774 macrophages. Plasmid pML31 encoding YopO$_{143\Delta 20-77}$-Cya (ΔCBD) was introduced into the multi-effector knockout mutant E40(pIML421) (called ΔHOPEMT) and into the translocator-deficient E40(pCNK4008) (called ΔHOPEMNB). Plasmid pCD10 encodes YopO$_{143}$-Cya (wt). Cellular cAMP levels were assayed after infection.

The CBDs of YopE and YopT are also aggregation-prone MLDs

The experiments described above demonstrate that SycO is required to mask the aggregation-prone MLD of YopO inside the bacterium. We wondered whether this hypothesis could apply to other class-I chaperones.

Like the CBD of YopO, residues 50–77 of YopE create the need for SycE (Boyd *et al*, 2000). Even more, residues 54–75 have been shown to represent an MLD for YopE (Krall *et al*, 2004). As shown in Figure 7A, YopE$_{90}$-EGFP expressed in HEK293T cells showed a peri-nuclear localization including the *cis*-Golgi. This does not mean that when YopE is injected by *Yersinia*, it would be associated to the Golgi, but it confirms that the CBD confers some membrane targeting properties to YopE, as shown earlier (Krall *et al*, 2004). It is thus likely that the CBD causes intrabacterial insolubility. We thus tested the intrabacterial solubility of YopE in the presence and absence of SycE. As shown in Figure 7D, SycE clearly increased the solubility of YopE. This result is in perfect agreement with previous data from Birtalan *et al* (2002) showing that binding of SycE rescues purified YopE from aggregation.

As YopT, like YopE, targets Rho GTPases (Zumbihl *et al*, 1999; Black and Bliska, 2000; Von Pawel-Rammingen *et al*, 2000; Shao *et al*, 2002) and also associates with host cell

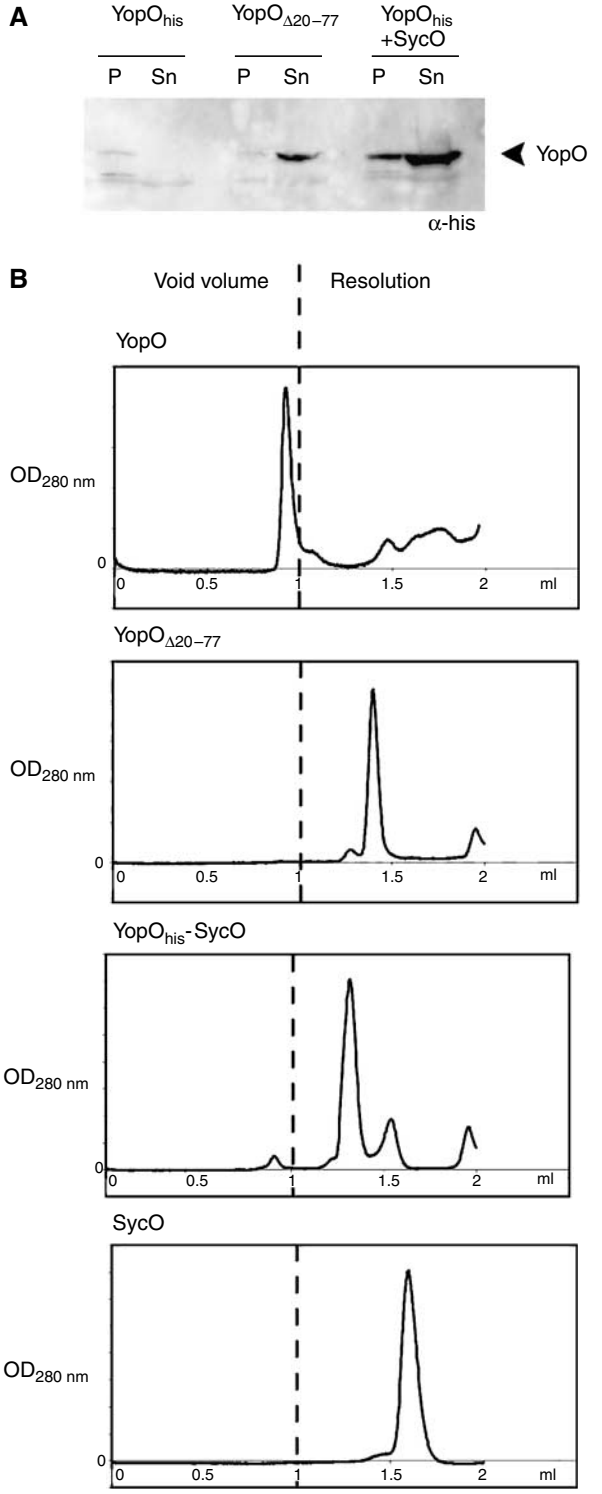

Figure 5 The CBD causes aggregation of YopO. (**A**) YopO$_{his}$, YopO$_{\Delta 20-77his}$ and YopO$_{his}$ + SycO were overexpressed in *E. coli* BL21 from plasmids pML7, pML8 and pML9, respectively. The cleared lysates (see Materials and methods) were centrifuged at 100 000 *g* to separate soluble (Sn) from insoluble (P) proteins and analyzed by Western blotting. (**B**) GST-YopO, GST-YopO$_{\Delta 20-77}$ and GST-YopO(SycO)$_2$ were synthesized in *E. coli* and purified as described in Supplementary data 4. After removal of the GST tag, the proteins were analyzed on a Superdex^{TM} 200 gel exclusion chromatography. Purified SycO (see Supplementary data 4) was analyzed in parallel.

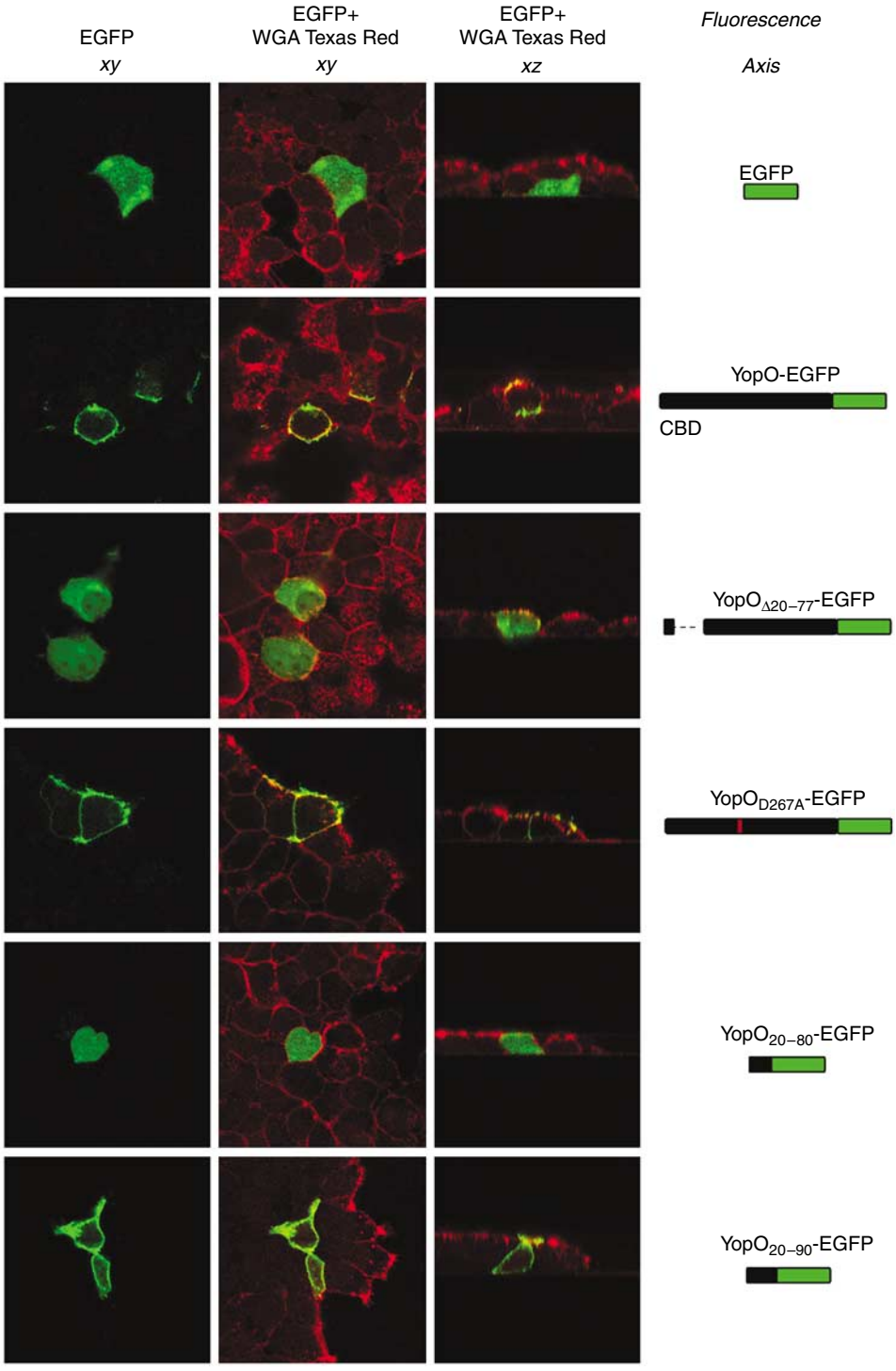

Figure 6 The CBD of YopO is an MLD. The different constructs drawn on the right panel were transfected in HEK293T cells. Cells were grown for 24 h, stained with Texas Red-conjugated wheat germ agglutinin, fixed and analyzed by confocal microscopy. The constructs were encoded by pML1 (YopO-EGFP), pML2 (YopO$_{\Delta 20-77}$-EGFP), pML3 (YopO$_{D267A}$-EGFP), pML26 (YopO$_{20-80}$-EGFP) and pML27 (YopO$_{20-90}$-EGFP).

membranes (Aepfelbacher *et al*, 2003), we would predict that the CBD of YopT is also an MLD. The CDB of YopT has been localized, by limited proteolysis, between residues 52 and 139 (Buttner *et al*, 2005). In order to test whether this CBD would also correspond to the MLD, we fused residues 1–124 of YopT to EGFP and expressed the hybrid in HEK293T cells. YopT$_{124}$-EGFP presented a punctuated distribution evoking either lysosomal association or aggregation (Figure 7B and C). Using LAMP1 as a lysosomal marker, we observed that YopT$_{124}$-EGFP did not colocalize with lysosomes, suggesting that YopT$_{124}$ was causing the aggregation of EGFP. We then tested whether the SycT chaperone would not influence the intrabacterial solubility of YopT. As shown in Figure 7D, this was indeed the case.

We fused the first 90 amino acids of YopH to EGFP, but no specific distribution was observed. EGFP was homo-

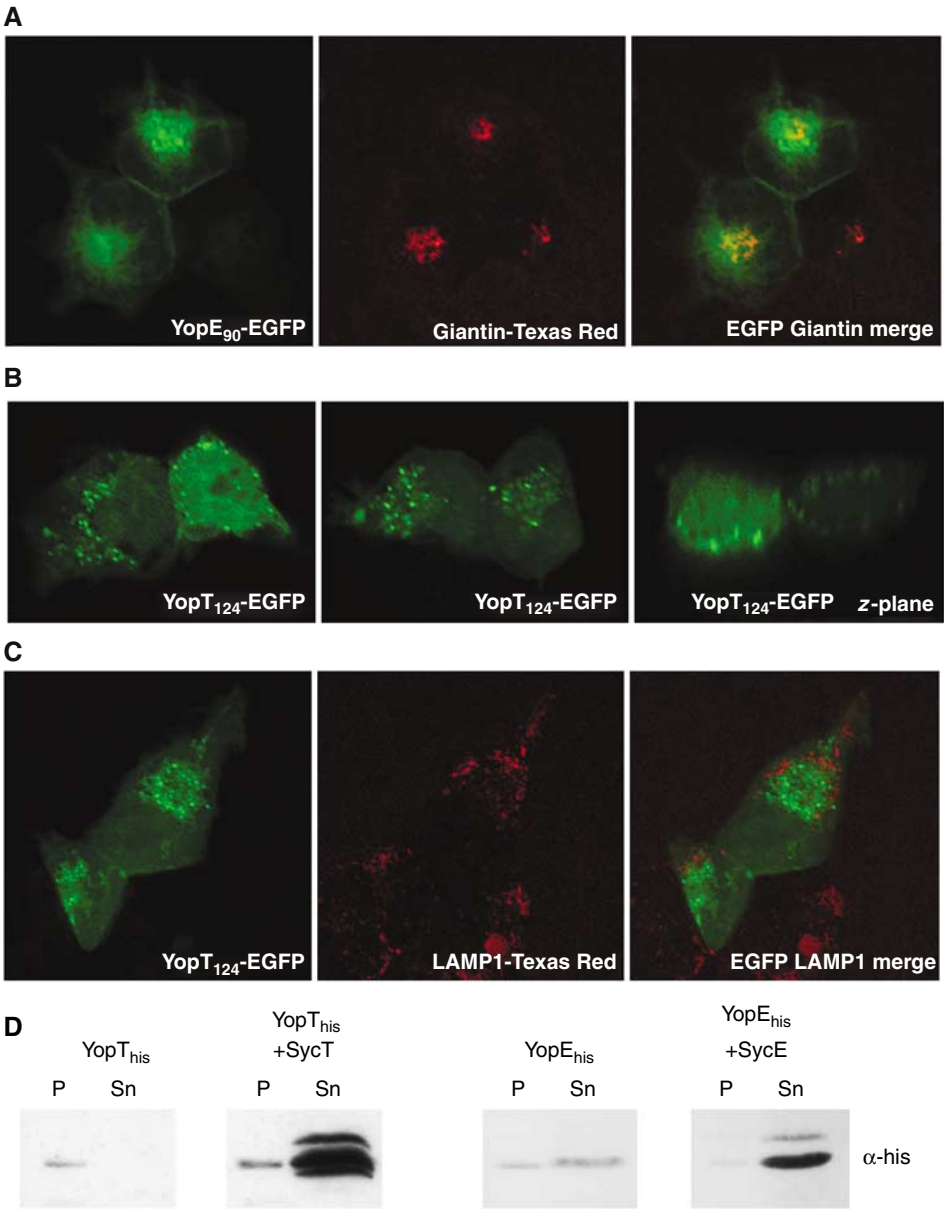

Figure 7 Role of the CBD of YopE and YopT. The fusions $YopE_{90}$-EGFP (**A**) and $YopT_{124}$-EGFP (**B, C**) were expressed in HEK293T cells after transfection of plasmids pSAM10 and pSAM12, respectively. Cells were grown 24 h, fixed, permeabilized and immunolabelled with α-Giantin (A) or α-LAMP1 (C). Pictures were acquired by confocal microscopy. (**D**) $YopE_{his}$ and $YopT_{his}$ encoded by pML33 and pML34 were expressed in *E. coli* BL21 with or without SycE or SycT encoded by plasmids pSAM13 or pSAM15, respectively. The cleared lysates (see Materials and methods) were centrifuged at 100 000 *g* to separate soluble (Sn) from insoluble (P) proteins and analyzed by Western blotting.

geneously distributed in the whole cell (data not shown). In addition, there was no clear difference in the solubility of YopH in the presence or absence of SycH.

Finally, we analyzed the cellular localization of YopP, for which no chaperone was described. The hybrid YopP-EGFP transfected into HEK293T cells appeared uniformly distributed to the cytosol (not shown).

In conclusion, we suggest that SycO, SycE and SycT cover an aggregation-prone MLD.

Discussion

In *Yersinia*, the chaperones of effector proteins are SycE (Wattiau and Cornelis, 1993; Birtalan and Ghosh, 2001), SycH (Wattiau *et al*, 1994; Phan *et al*, 2004) and SycT (Iriarte and Cornelis, 1998; Buttner *et al*, 2005; Locher *et al*, 2005). SycO described in this paper shares all the properties of these Syc proteins and clearly represents a new member of this group. However, its phenotype is somewhat more subtle and this explains why it is discovered more than 10 years later than the others. At first sight, SycO does not appear to be required for *in vitro* secretion of YopO but it is required for translocation of YopO into cells. If chaperones are necessary for export of effector molecules that are stored in the cytoplasm, but not for export of those synthesized while the secretion apparatus is active, as demonstrated by Parsot and co-workers (Page *et al*, 2002) for Spa15, then our results indicate that translocation of YopO into cells is essentially post-translational. In contrast, *in vitro* secretion of YopO, induced by Ca^{2+} chelation, could be also co-translational.

Having shown that SycO is a new member of the group of *Yersinia* effector chaperones, we tried to address its function, taking into consideration the different functions already proposed for the proteins of this group. We observed that binding of SycO to YopO does not prevent the activity of the kinase domain of YopO, implying that the catalytic domain (residues 160–400) and the actin-binding domain (residues 709–729) (Juris *et al*, 2000) are folded. Thus, SycO does not maintain YopO in a completely unfolded secretion-competent state. This observation is in perfect agreement with the observation of Birtalan *et al* (2002) that SycE does not prevent the GAP activity of YopE and with the observation of Akeda and Galan (2005) that SicP does not prevent the tyrosine phosphatase activity of SptP, revising an earlier interpretation of Stebbins and Galan (2001). For SycO, we thus rule out that its function is to maintain YopO in an unfolded state, which also implies that the injectisome can unfold its substrates during export, as was clearly demonstrated for SptP by Akeda and Galan (2005).

However, we observed that SycO keeps YopO soluble in the cytosol of *Yersinia* before export and that it is precisely the SycO-binding domain that makes YopO insoluble. Thus, the presence of the CBD creates the need for the chaperone. As pointed out by Birtalan *et al* (2002), it is unlikely that chaperones exist simply to mask an aggregation-prone effector region whose only function is chaperone binding. We thus looked for a function of this aggregation-prone domain and we found that it was an MLD. A function of SycO is thus to hide the aggregation-prone MLD of YopO while YopO is in the bacterium.

Would this new function apply to other class-I chaperones? Previous data from Krall *et al* (2004) and data presented here show that it also applies to YopE. It probably also applies to YopT because the CBD causes aggregation of transfected hybrid proteins in the host cell. However, it should still be demonstrated that this domain targets YopT to the membrane when YopT is injected rather than transfected. We also tried to extend the hypothesis to YopH, which targets focal adhesions (Persson *et al*, 1999) and another membrane-associated complex (Black *et al*, 2000). We did not observe any membrane localization of transfected $YopH_{90}$-EGFP and any change in the intrabacterial solubility of YopH with or without SycH. However, for YopH, the situation could be more complex as the CBD already partially overlaps with the phospho-tyrosine recognition domain (Montagna *et al*, 2001). Moreover, different domains of YopH including its CBD have been shown to interact with the Fyn binding protein in macrophages (Yuan *et al*, 2005). Thus, the CBD of YopH might not be a membrane targeting domain, it is nevertheless an intracellular targeting domain. In good agreement with our hypothesis about class-I chaperones, YopM (Benabdillah *et al*, 2004) and YopP, which do not have a chaperone, do not localize to membranes. In *Yersinia*, there is thus a correlation between the presence of a chaperone and an activity of the effector at the membrane of the target cell.

Would this new hypothesis about effector chaperones and MLDs also apply to effectors of other T3S systems? The hypothesis predicts that the effectors that have a chaperone would act at the membrane of the host cell. Among the best-characterized class-I chaperones, we find SicP (Fu and Galan, 1998) and SigE (Darwin *et al*, 2001) from *Salmonella enterica,* CesT from enteropathogenic *E. coli* (Elliott *et al*, 1999) and Spa15 from *Shigella* (Page *et al*, 2002). SptP, the partner of SicP, is a GAP for Rho (Fu and Galan, 1999) and has been shown to localize at the plasma membrane of infected cells (Cain *et al*, 2004); SigD/SopB, the partner of SigE, is an inositol phosphatase (Norris *et al*, 1998), which was shown to be membrane-associated (Marcus *et al*, 2002; Cain *et al*, 2004); Tir, the partner of CesT, is the membrane-associated intimin receptor (Kenny *et al*, 1997); and finally, Spa15 is, among others, the chaperone of IpaA, which contributes to *Shigella* entry by binding the focal adhesion protein vinculin (Tran Van Nhieu *et al*, 2000). Thus, all these effectors have a chaperone of the SycE family, and are targeted to the membrane. Furthermore, the *Salmonella* effectors SopE, SopE2 and SipA, which all share the InvB chaperone (Bronstein *et al*, 2000; Ehrbar *et al*, 2003), have also been shown to localize at the membrane of infected cells (Cain *et al*, 2004).

Does this new function for class-I chaperones exclude any targeting function? Certainly not, as the CBD and SycO are required for efficient delivery of YopO into cells by wt *Yersinia* bacteria. This situation is reminiscent of YopE. Indeed, YopE deprived of its CBD cannot be delivered by wt bacteria but it can be delivered by mutant bacteria deprived of all the other effectors (Boyd *et al*, 2000). Chaperones bound to the effector thus improve targeting to the secretion apparatus, either by acting as a three-dimensional secretion signal, as suggested for SycE (Birtalan *et al*, 2002), or simply by presenting the secretion signal. The presence of the chaperone could also favor the interaction with the ATPase, and so facilitate the unfolding of the effector (Akeda and Galan, 2005). Our data do not allow to discriminate between these hypotheses.

We thus suggest that the class-I chaperones have two functions: (i) cover an aggregation-prone MLD and (ii) facilitate export. Which one of these two functions would have appeared first? As some effectors seem not to have a chaperone (YopM and YopP in *Yersinia*), we would speculate that the primary function of effector chaperones is to cover an MLD and that targeting of effector chaperone complexes has been gradually optimized by evolution. In support of this view, YopO and YopE deprived of their CBD can be translocated by multi-effector knockout mutant bacteria. CBDs and chaperones thus evolved to serve two different essential functions, one inside the bacterium and one inside the host cell.

Materials and methods

Bacterial strains, plasmids and genetic constructions

Y. enterocolitica E40 (Sory and Cornelis, 1994) and W22703 (Cornelis *et al*, 1986), both from serotype 0:9, were used for T3S experiments. *E. coli* BL21 was used for protein expression and *E. coli* Top10 was used for plasmid amplification and cloning. Plasmids are listed in Table I. Oligonucleotides labelled with * in Table I were used to delete or mutate domains by inverse polymerase chain reaction, using the *Pfu* turbo polymerase (Stratagene). Every construct was sequenced using 3100-*Avant* genetic analyzer (ABI Prism). Oligonucleotides are listed in Supplementary Table S1.

Induction of type III secretion by low Ca^{2+}

This was carried out as described earlier (Agrain *et al*, 2005).

Standard protein purification and analysis protocols

See Supplementary data 3.

Ultracentrifugation of crude extracts

$YopO_{his}$, $YopO_{\Delta 20-77his}$ and SycO coexpressed with $YopO_{his}$ were produced in *E. coli* BL21 from the pET22 vector using plasmids pML7, pML8 and pML9. Bacteria were grown overnight at 37°C,

Table I List of the plasmids used in this work

pYV derivatives

Plasmids	Characteristics	References
pYVe227	wt pYV plasmid from *Y. enterocolitica* W227	Cornelis *et al* (1986)
pYV40	wt pYV plasmid from *Y. enterocolitica* E40	Sory *et al* (1995)
pML4001	pYV40: *sycO*	This study
pAB406	pYV40: $yopO_{\Delta 65-558}$	Mills *et al* (1997)
pSW2276	pYV40: $yscN_{\Delta 169-177}$	Woestyn *et al* (1994)
pIML421(ΔHOPEMT)	pYV40: $yopH_{\Delta 1-352}$, $yopO_{\Delta 65-558}$, $yopP_{23}$, $yopE_{21}$, $yopM_{23}$, $yopT_{135}$	Iriarte and Cornelis (1998)
pCNK4008 (ΔHOPEMNB)	pYV40: $yopH_{\Delta 1-352}$, $yopO_{\Delta 65-558}$, $yopP_{23}$, $yopE_{21}$, $yopM_{23}$, $yopN_{45}$, $yopB_{\Delta 89-217}$	Neyt and Cornelis (1999)

Clones

Plasmids	Characteristics	Derivation and oligonucleotides used	References
pCD10	pTM100::$yopO_{143}$-$cyaA'_{(sycE\ promoter)}$		Unpublished
pCD11	pTM100::$yopO_{77}$-$cyaA'_{(sycE\ promoter)}$	Deletion of the *Mun*I/*Hin*dIII fragment in pCD10	Unpublished
pISO56	pBBR1 MCS2::$yopO_{flag(yopE\ promoter)}$	Flag-tag insertion in pMAF60 *(3688/3710)	This study
pMAF60	pBBR1 MCS2::$yopO_{(yopE\ promoter)}$	Cloning of *yopO* from pYOB2 3118/3119	This study
pML1	pEGFP(N)::*yopO*	Cloning of *yopO* 3394/3395	This study
pML2	pEGFP(N)::$yopO_{\Delta 20-77}$	Deletion from pML1 *(3396/3397)	This study
pML3	pEGFP(N)::$yopO_{D267A}$	Mutation on pML1 *(3484/3485)	This study
pML4	pBBR1 MCS2::*sycO*	Cloning of *sycO* 3266/3271	This study
pML7	pET22::$yopO_{his}$	Cloning of *yopO* 3523/3525	This study
pML8	pET22::$yopO_{\Delta 20-77\ his}$	Deletion from pML7 *(3396/3397)	This study
pML9	pET22::*sycO-yopO*$_{his}$	Cloning of *sycO-yopO* 3524/3525	This study
pML10	pGEX-6p-1::*yopO*	Cloning of *yopO* 3698/3699	This study
pML11	pGEX-6p-1::$yopO_{\Delta 20-77}$	Deletion from pML10 *(3396/3397)	This study
pML12	pGEX-6p-1::$yopO_{D267A}$	Mutation on pML10 *(3484/3485)	This study
pML13	pGEX-6p-1::$yopO_{200}$	Cloning of $yopO_{200}$ 3523/3908	This study
pML14	pBAD::*sycO*	Cloning of *sycO* 3268/3250	This study
pML15	pCDF::*sycO*	Cloning of *sycO* 3268/3271	This study
pML16	pBBR1 MCS2::$yopO_{\Delta 20-77\ flag\ (yopE\ promoter)}$	Deletion from pISO56*(3396/3397)	This study
pML17	pGEX-6p-1::$yopO_{20-77}$	Cloning of $yopO_{20-77}$ 4015/4028	This study
pML18	pKS::−250/*sycO*/+250	Cloning of −250/*sycO*/+250 3266/3267	This study
pML19	pKS::−250/Δ*sycO*/+250	Deletion of *sycO* from pML18 *(3272/3273)	This study
pML26	pEGFP(N)::$yopO_{20-80}$	Cloning of $yopO_{20-80}$ 4134/4079	This study
pML27	pEGFP(N)::$yopO_{20-90}$	Cloning of $yopO_{20-90}$ 4134/4080	This study
pML31	pBBR1 MCS2::$yopO_{143\Delta 20-77}$-$cyaA'_{(sycE\ promoter)}$	Deletion from pCD10*(3396/3397)	This study
pML33	pET22::$yopE_{his}$	Cloning of *yopE* 4306/4309	This study
pML34	pET22::$yopT_{his}$	Cloning of *yopT* 4307/4310	This study
pMSLE20	pTM100::$yopE_{20}$-$cyaA'$		Sory *et al* (1995)
pSAM10	pEGFP(N)::$yopE_{1-90}$	Cloning of $yopE_{1-90}$ 4293/4294	This study
pSAM12	pEGFP(N)::$yopT_{1-124}$	Cloning of $yopT_{1-124}$ 4297/4298	This study
pSAM13	pCDF::*sycE*	Cloning of *sycE* 4301/4302	This study
pSAM15	pCDF::*sycT*	Cloning of *sycT* 4299/4300	This study
pYOB2	pCNR26::$yopO_{(yopE\ promoter)}$	Cloning of *yopO* 471/473	This study

Cloning vectors

Plasmids	Characteristics	References
pBADmyc-his A	pBAD promoter, high copy	Invitrogen
pBBR1-MCS2	Medium–low copy	Kovach *et al* (1995)
pBluescript II KS (+)		Stratagene
pCDF-Duet	T7 promoter	Novagen
pCNR26	*yopE* promoter	Sarker *et al* (1998)
pEGFP-N1	CMV IE promoter	BD Biosciences Clontech
pET22	T7 promoter, *lac* operator, high copy	Novagen
pGEX-6p-1	p*Tac* promoter	Amersham
pTM100	pACYC184-*oriT*	Michiels and Cornelis (1991)

Suicide vectors, mutator

Plasmids	Characteristics	References
pKNG101	*ori* R6K, *mob* RK2, *strAB*, *sacBR*	Kaniga *et al* (1991)
pML20	pKNG101::−250/Δ*sycO*/+250	This study

Oligonucleotides labelled with * were used to delete or mutate domains by inverse polymerase chain reaction using *Pfu* turbo polymerase (Stratagene).

diluted to an OD_{600} of 0.1 in LB with ampicillin 100 μg ml^{-1} and incubated at 37°C until they reached an OD_{600} of 0.6. Then, the expression of the proteins was induced by adding 0.125 mM IPTG and the cultures were incubated at room temperature (RT) with shaking for 3 h. Cells were harvested by centrifugation, resuspended in PBS containing Triton X-100 0.1% (v/v), protease inhibitors (Complete mini, Roche) and lysed by using a French press. The lysate was spun for 30 min at 6000 *g* to eliminate unbroken cells and debris. The supernatant was centrifuged for 1 h 30 min at 100 000 *g* to separate soluble compounds (supernatant) from insoluble ones (pellet).

Kinase assay

Proteins were purified as described in Supplementary data 4. YopO and $YopO_{D267A}$ were eluted together with GST in order to improve their solubility. The purified YopO-$(SycO)_2$ complex used for the kinase assay was previously run on gel filtration in order to use only proteins associated in a complex. Kinase assays were performed using 5 μg of purified proteins in the kinase reaction buffer: PBS, 20 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP and 2 μCi [γ-^{32}P]ATP, ±0.1 μg of purified G-actin (Pierce). The reaction was incubated at 30°C for 30 min and stopped by addition of SDS–PAGE loading buffer. The kinase reactions were loaded on SDS–PAGE gel. The autoradiography was obtained on Phosphor Screen (Molecular Dynamics).

Adenylate cyclase reporter translocation assay

J774 macrophages were grown in RPMI 1640 medium (Gibco BRL), supplemented with 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.0004% (v/v) β-mercaptoethanol (Sigma) and 10% (v/v) fetal bovine serum (Invitrogen). Adenylate cyclase reporter translocation assays were carried out as described earlier (Sory and Cornelis, 1994). All experiments were performed in triplicate.

Transfection and fluorescence microscopy

HEK293T cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL), supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 1 mM sodium pyruvate (Invitrogen). Coverslips (Huber & Co) were coated with poly-lysine (BD Diagnostic System), and 10^4 cells well^{-1} were seeded in 500 μl of media. The next day, cells were transfected following the calcium phosphate procedure. Cells were grown for 24 h, stained with 10 μg ml^{-1} wheat germ agglutinin Texas Red®-X conjugate (Molecular Probes) for 7 min at RT and fixed for 20 min in 3% paraformaldehyde in PBS. For Giantin and LAMP1 staining, cells were permeabilized with 0.1% saponin in PBS containing 3% BSA, incubated during 40 min with α-Giantin (G1/133) or α-LAMP1 (G1/139) (Axxora), washed and incubated for 30 min with a Texas Red®-coupled secondary antibody (Molecular Probes). Slides were examined with a Leica TCS SP confocal microscope. Pictures were processed with the Leica confocal software version 2.5.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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